Example 14: Direct Recognition of Duplex Oligonucleotides Without Dehybridization, Using Assembly of Nanoparticle-Oligonucleotide Conjugates

In the previous Examples, double-stranded targets were dehybridized by heating to generate single strands which interacted with single-stranded oligonucleotide probes bound to nanoparticles. The present example demonstrates that in cases where triple-stranded complexes can form, double-stranded oligonucleotide sequences can be recognized by the nanoparticle probes without prior dehybridization of the target.

Tests were carried out with two different systems-- polyA:polyU and dA_{40}:dT_{40} -- by adding 1 μL of a solution containing 0.8 A_{260} Units of the target duplex in 100 μL of buffer (0.1 M NaCl, 10 mM phosphate, pH 7.0) to 100 μL of a colloidal solution of Au-sdT_{20} nanoparticle-oligonucleotide conjugate (~10 nM in particles; see Example 11) in 0.3 M NaCl, 10 mM phosphate buffer at pH 7.0. Subsequent quick freezing by immersing the tube in a Dry Ice/isopropyl alcohol bath and thawing by removing the tube from the bath and letting it stand at room temperature (22°C), followed by spotting 3 μL of the solution on a C18 TLC plate, afforded a blue spot characteristic of hybridization and aggregation of the nanoparticles.

The rationale for this test is that the nanoparticle probes (bearing pyrimidine oligonucleotides in this example) bind in a sequence specific manner at purine oligonucleotide/pyrimidine oligonucleotide sites along the duplex target. Since many binding sites are available on each double stranded entity, the binding leads to formation of an aggregate of nanoparticles. The results show that this assay, based on formation of triple-stranded complexes involving the nanoparticle probes, works both for oligoribo- and oligodeoxyribonucleotide double-stranded targets.

Example 15: Assay Employing Both Fluorescence And Colorimetric Detection

All hybridization experiments were performed in a 0.3 M NaCl, 10 mM phosphate, pH 7.0, buffer solution. AcetatePlus™ filtration membranes (0.45 μm) were purchased from Micron Separations Inc., Westboro, MA. Alkylamine-functionalized latex microspheres (3.1

μm) were purchased from Bangs Laboratories, Fishers IN. Fluorophore-labeled oligonucleotides functionalized with alklylamino groups at the 3'-terminus were synthesized using standard phosphoramidite chemistry (Eckstein, ed., in *Oligonucleotides and Analogues*, 1st ed., Oxford University, New York, N.Y. 1991) with an Amino-Modifier C7 CPG solid support (Glen Research) and a 5'-fluorescein phosphoramidite (6-FAM, Glen Research) on an Expedite 8909 synthesizer and were purified by reverse phase HPLC. They were attached to the amine-functionalized latex microspheres by means of diisothiocyanate coupling to yield a dithiourea linkage as described in Charreyre et al., *Langmuir*, **13**, 3103-3110 (1997). Briefly, a DMF solution of a one thousand fold excess of 1,4-phenylene diisothiocyanate was added to an aqueous borate buffer solution (0.1 M, pH 9.3) of the amino-modified oligonucleotide. After several hours, the excess 1,4-phenylene diisothiocyanate was extracted with butanol and the aqueous solution lyophilized. The activated oligonucleotides were redissolved in borate buffer and reacted with the amino-functionalized latex microspheres in a carbonate buffer (0.1 M, pH 9.3, 1 M NaCl). After 12 hrs, the particles were isolated by centrifugation and washed three times with buffered saline solution (0.3 M NaCl, 10 mM phosphate pH 7.0). The 5'-oligonucleotide-modified gold nanoparticle probes were prepared as described in Example 3.

The target oligonucleotide (1-5 μl, 3 nM) was added to 3 μl of fluorophore-labeled oligonucleotide-modified latex microsphere probe solution (3.1 μm; 100 fM). After 5 minutes, 3 μl of the 5' oligonucleotide-modified gold nanoparticle probe solution (13 nm; 8 nM) were added to the solution containing the target and latex microsphere probes. Upon standing for an additional 10 minutes, the solution containing both probes and target was vacuum-filtered through the AcetatePlus membrane. The membrane retained the relatively large latex particles and allowed any non-hybridized gold nanoparticle probes to pass through. In the presence of a sufficient concentration of target, the latex microspheres and the gold nanoparticles hybridized with the target, and a red spot was observed on the membrane (positive result). A control experiment was always carried out where the aliquot of solution containing the target oligonucleotide was replaced by an equal volume of water.

In this case, a white spot was left on the membrane (negative result). For a 24-base-pair model system, using the unaided eye, 3 femtomoles of target oligonucleotide could be detected colorimetrically.

A double-stranded target oligonucleotide (1-5 μl, 20 nM), 3 μl of a solution of fluorophore-labeled- oligonucleotide-latex microspheres (3.1 μm; 100 fM) and 3 μl of a solution of 5'-oligonucleotide-gold nanoparticles (13 nm; 8 nM) were combined and heated to 100 °C for 3 minutes. Then, the solution was immediately frozen by immersing the reaction vessel containing it in a liquid N_2 bath for 3 minutes. This solution was then thawed at room temperature and filtered as described above. For a 24-base pair model system, using the unaided eye, 20 femtomoles of duplex target oligonucleotide could be detected colorimetrically.

When monitored by fluorescence, the detection method described above proved to be difficult due to background fluorescence from the membrane. This problem was overcome by "washing" the latex microspheres by centrifugation to remove excess gold nanoparticle probes before spotting an aliquot on a reverse-phase TLC plate. The hybridization experiments were performed as described above. After hybridization was effected between the probes and target, 10 μl of buffer were added to the solution, which was subsequently centrifuged at 10,000 x g for 2 minutes. The supernatant was removed, and 5 μl of buffer were added to help resuspend the precipitate. A 3 μl aliquot was then spotted on a reverse-phase TLC plate. For both single-stranded and duplex target oligonucleotides, 25 femtomoles could be detected colorimetrically by the naked eye. Fluorescent spots could be visualized by the naked eye with a hand-held UV-lamp until the target amount in the 3 μl aliquot used to form the spot was as low as 50 femtomoles. It is believed that optimization of this system will allow for detection of even lower amounts of target nucleic acid.

Example 16: Assays Employing Silver Staining

DNA hybridization tests on oligonucleotide-modified substrates are commonly used to detect the presence of specific DNA sequences in solution. The developing promise of